

A copper sulfate and hydroxylysine treatment regimen for enhancing collagen cross-linking and biomechanical properties in engineered neocartilage

Eleftherios A. Makris,^{*,†} Regina F. MacBarb,^{*} Donald J. Responde,^{*} Jerry C. Hu,^{*} and Kyriacos A. Athanasiou^{*,1}

^{*}Department of Biomedical Engineering, University of California–Davis, Davis, CA USA; and

[†]Department of Orthopedic Surgery and Musculoskeletal Trauma, University of Thessaly (Biomed), Larisa, Greece

ABSTRACT The objective of this study was to improve the biomechanical properties of engineered neotissues through promoting the development of collagen cross-links. It was hypothesized that supplementing medium with copper sulfate and the amino acid hydroxylysine would enhance the activity of lysyl oxidase enzyme to form collagen cross-links, increasing the strength and integrity of the neotissue. Neocartilage constructs were generated using a scaffoldless, self-assembling process and treated with copper sulfate and hydroxylysine, either alone or in combination, following a 2-factor, full-factorial study design. Following a 6-wk culture period, the biomechanical and biochemical properties of the constructs were measured. Results found copper sulfate to significantly increase pyridinoline (PYR) cross-links in all copper sulfate-containing groups over controls. When copper sulfate and hydroxylysine were combined, the result was synergistic, with a 10-fold increase in PYR content over controls. This increase in PYR cross-links manifested in a 3.3-fold significant increase in the tensile properties of the copper sulfate + hydroxylysine group. In addition, an 123% increase over control values was detected in the copper sulfate group in terms of the aggregate modulus. These data elucidate the role of copper sulfate and hydroxylysine toward improving the biomechanical properties of neotissues through collagen cross-linking enhancement.—Makris, E. A., MacBarb, R. F., Responde, D. J., Hu, J. C., Athanasiou, K. A. A copper sulfate and hydroxylysine treatment regimen for enhancing collagen cross-linking and biomechanical properties in engineered neocartilage. *FASEB J.* 27, 000–000 (2013). www.fasebj.org

Key Words: articular cartilage regeneration • covalent intermolecular bonds • scaffoldless self-assembly

ARTHRITIS IS THE SECOND most frequent chronic disease in the United States. Currently affecting 46.4 million people and costing the United States more than \$128 billion per annum, the economic burden of this disease is tremendous and growing (1). The most common form of arthritis, known as osteoarthritis (OA), is characteristic of joint cartilage degeneration (2). Due to the inability of articular cartilage to self-repair, there are currently no clinical options for treating OA. The ability to generate engineered articular cartilage *in vitro*, therefore, holds much promise as an effective reparative treatment for OA. Many advances have been made in articular cartilage tissue engineering strategies toward developing a biomechanically robust repair tissue able to integrate with the surrounding healthy cartilage. Though much work remains to be done to engineer clinically relevant repair cartilage, tissue engineering approaches offer new hope toward bringing a degenerated joint back to an uncompromised state.

Current tissue engineering approaches for generating articular cartilage typically involve a combination of cells, signals (*e.g.*, growth factors, mechanical stimulation), and scaffolds. Deviating from the typical tissue engineering paradigm, our group has developed a self-assembly method in which we forgo the use of a scaffold. By culturing articular chondrocytes at high densities in 3-dimensional cultures using nonadherent agarose wells, we are able to form robust neocartilage in a manner akin to native cartilage morphogenesis (3–5). This process is advantageous in that it is not affected by the inherent problems associated with scaffolds, such as biodegradability, stress-shielding, and hindering of cell-to-cell communication. However, as with scaffold-based tissue engineering approaches, self-assembled articular cartilage often fails to match the high mechanical strength of native tissue, leaving it incapable of with-

Abbreviations: Col/WW, collagen content normalized to construct wet weight; DMEM, Dulbecco's modified Eagle medium; ECM, extracellular matrix; E_y , Young's modulus; GAG, glycosaminoglycan; GAG/WW, GAG content normalized to construct wet weight; H_A , aggregate modulus; HPLC, high performance liquid chromatography; LOX, lysyl oxidase; OA, osteoarthritis; PSF, penicillin/streptomycin/fungizone; PYR, pyridinoline; PYR/Col, pyridinoline content normalized to construct collagen content; PYR/WW, pyridinoline content normalized to construct wet weight; UTS, ultimate tensile strength

¹ Correspondence: Department of Biomedical Engineering, University of California Davis, One Shields Ave, Davis, CA 95616, USA. E-mail: athanasiou@ucdavis.edu

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standing the complex loading scheme of an articular joint. These inferior properties mainly manifest in the tensile properties of engineered neotissue; targeted efforts to increase the tensile properties of engineered articular cartilage are, therefore, crucial to creating tissues with clinical impact.

The ability of articular joints to withstand high loads is mainly due to the load adsorbing and distributing properties of articular cartilage. With structure and function being intimately linked, a direct tie between mechanical integrity and both extracellular matrix (ECM) composition and structure has been observed. In the case of articular cartilage, the typical structure-function paradigm links the presence of an organized collagen network with the tissue's tensile strength, while compressive stiffness has been related to glycosaminoglycan (GAG) distribution within the tissue (6). However, recent investigations in both native and engineered articular cartilage have found this paradigm to shift. Such studies have reported that the collagen network significantly affects the compressive properties of articular cartilage (7, 8). Specifically, a better correlation between the compressive modulus and the collagen content has been described (9). Similarly, another study examining the correlation between the biomechanical and biochemical properties of superficial zone articular cartilage reported a correlation among the compressive modulus and both collagen and GAG, with R^2 values of 0.36 and 0.24, respectively (10). To explain this finding, it was hypothesized that increased collagen content may decrease the extrafibrillar volume and create a higher effective fixed charge density, which could increase tissue compressive properties (11).

The structure-function relationships related to collagen cross-linking have also been studied, although not as extensively as the effects of GAG and collagen. Pyridinoline (PYR) cross-links have been correlated with the tensile properties of native articular cartilage (12). Likewise, a significant correlation among collagen cross-links and compressive properties of native articular cartilage has also been reported (9). Such studies indicate that the specific organization of the collagen architecture, including fiber density, diameter, orientation, and degree of cross-linking, all play a significant role in determining the mechanical integrity of both native and engineered tissues. These additional structure-function relationships will need to be elucidated to fully understand the biomechanics of cartilage.

Although current attempts to increase the tensile properties of engineered articular cartilage frequently focus on collagen content and alignment, increasing interfibrillar collagen cross-links may provide an additional means to increase the tensile properties of neocartilage. While several investigations have characterized the nature of collagen cross-linking in native tissue, only a few studies have focused on the type, amount, and influence of collagen cross-linking in engineered tissues (13). For instance, it has been shown that chondrocytes cultured on polyglycolic acid scaffolds or suspended within alginate beads produce collagen cross-links; however, reported values were far from those of native articular cartilage (14, 15). Furthermore, another study has demonstrated that colla-

gen networks in engineered cartilage require proper cross-linking to stabilize the matrix and to provide it with mechanical strength (16). Taken together, these studies illuminate the crucial role of cross-links in the stabilization and mechanical support of the collagen network within both native and engineered cartilages. Methods must therefore be investigated to induce the formation of collagen cross-links within engineered neocartilage to generate tissues with functional properties matching those of native tissue.

In native cartilage, the formation of lysine-derived, covalent PYR cross-links is dependent on the enzyme lysyl oxidase (LOX). To investigate the importance of LOX in articular cartilage, explants were treated with β -aminopropionitrile, a potent inhibitor of extracellular LOX activity. This treatment resulted in disorganization of the collagen architecture within the explants as well as a significant decrease in the biomechanical properties of the tissue (17). Such work highlights the importance of LOX-mediated collagen cross-links on the functional integrity of articular cartilage. LOX is a cuproenzyme, meaning its activity is dependent on the presence of copper (18). Once activated by copper, LOX is able to modify the amino acids lysine and hydroxylysine into PYR cross-links, the most abundant type of cross-link in native articular cartilage (Fig. 1 and refs. 19–21). It is important to note that copper sulfate is not a common ingredient in culture mediums, leaving LOX in its inactive form in *in vitro* culture. Therefore, copper sulfate supplementation may be a powerful means to enhance the amount of collagen cross-links and, therefore, improve the functional properties of engineered tissues.

Previous work on LOX has shown this enzyme to act extracellularly, requiring collagen fibrils in a quarter-staggered array as a substrate to generate cross-links (22, 23). Furthermore, the proper aldehyde precursors for LOX must be present in the immature collagen fibrils, including both lysine and hydroxylysine (Fig. 1). While lysine is present as an essential component in culture medium, hydroxylysine can be formed only when the enzyme lysyl hydroxylase catalyzes the conversion of lysine into its hydroxylated form. This is crucial to generate mature PYR cross-links (19, 20). Despite its importance in the development of a functionally mature collagen matrix, this essential collagen cross-link precursor is currently not used in cartilage tissue engineering, meriting studies to investigate its potential for increasing interfibrillar collagen cross-linking in engineered articular cartilage.

The overall objective of this study was to enhance collagen cross-linking in engineered articular cartilage to improve the biomechanical properties toward those of native tissue. Articular cartilage constructs were generated using the self-assembly process and supplemented with copper sulfate and hydroxylysine either alone or in combination following a full-factorial study design. Overall, it was hypothesized that chondrogenic medium supplemented with copper sulfate and hydroxylysine would additively or synergistically enhance the amount of LOX-mediated collagen cross-links, thus increasing the tensile strength of the engineered neotissue.

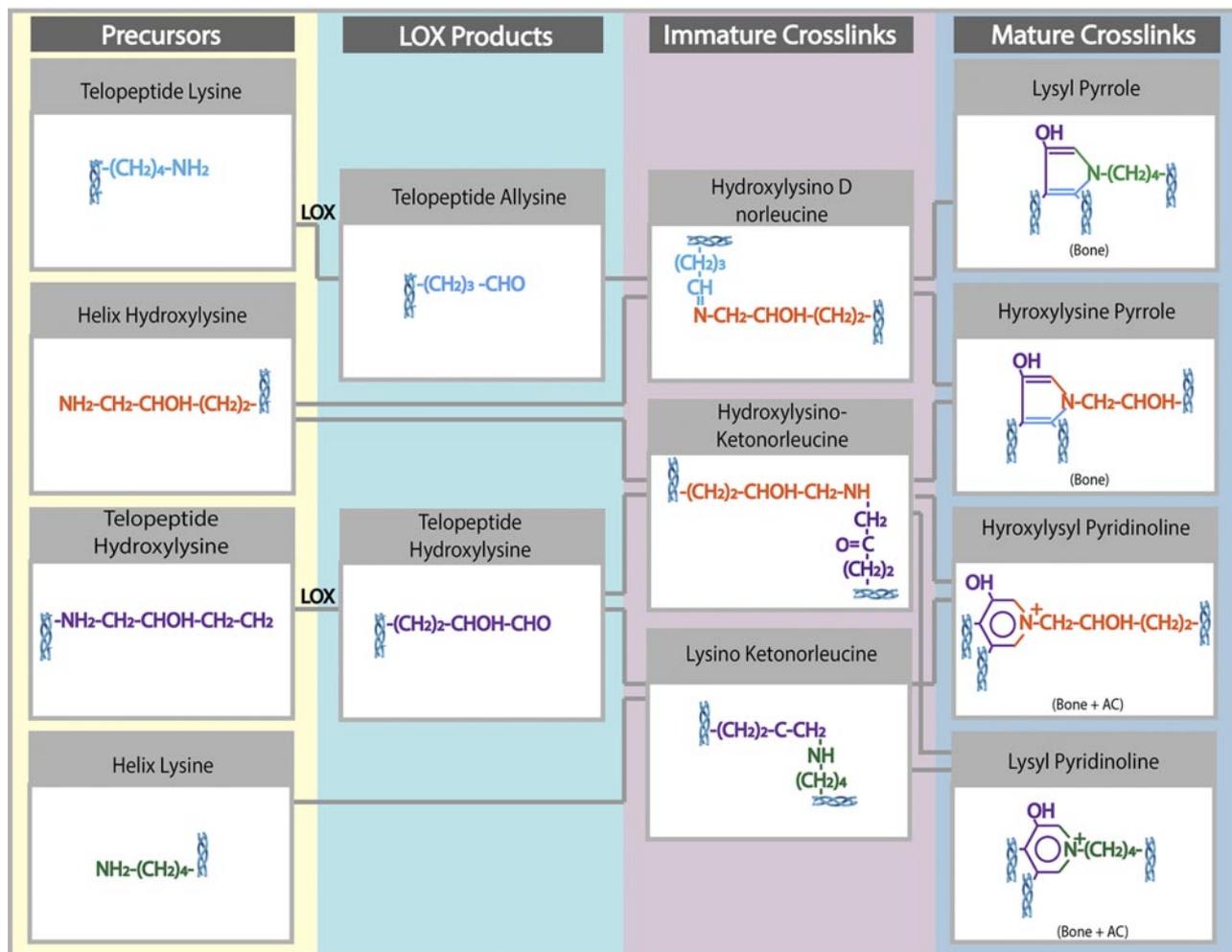


Figure 1. Molecular pathway of collagen cross-links in musculoskeletal tissues initiated by the extracellular enzyme LOX. Triple helix and telopeptide lysines are tracked by color, from initial to mature cross-links. Bone contains both PYR and pyrrole collagen cross-links, while articular cartilage features only PYR cross-links. Adapted from Eyre *et al.* (20).

MATERIALS AND METHODS

Harvest, isolation, and self-assembly

Juvenile bovine knee joints were obtained (Research 87, Boston, MA, USA) and harvested for the articular cartilage. Articular cartilage was minced from the condyles and patellar groove, and digested in a base medium of Dulbecco's modified Eagle medium (DMEM) supplemented with 1% penicillin/streptomycin/fungizone (PSF) and 2% collagenase (Worthington, Lakewood, NJ, USA) for 18 h. Following digestion, chondrocytes were isolated and purified *via* filtration and centrifugation, and then stored in base medium containing 20% fetal bovine serum and 10% dimethyl sulfoxide in liquid nitrogen until needed for seeding.

For self-assembly, chondrocytes were defrosted, counted, and seeded into 5-mm-diameter, 2% agarose wells at 5×10^6 cells/well as described previously (4). For the first week of culture, constructs were fed 500 μ l of chondrogenic medium consisting of DMEM, 1% PSF, 1% nonessential amino acids, 100 nM dexamethasone (Sigma-Aldrich, St. Louis, MO, USA), 1% ITS+ (BD Scientific, Franklin Lakes, NJ, USA), 40 μ g/ml L-proline, 50 μ g/ml ascorbate-2-phosphate, and 100 μ g/ml sodium pyruvate (Fisher Scientific, Pittsburgh, PA, USA). At d 7, the constructs were moved from the agarose molds into nonadherent tissue culture plates. Constructs were fed 1 ml of chondrogenic medium until the end of the 6-wk culture period.

Treatments

In this study, the effect of both copper sulfate (Sigma-Aldrich) and hydroxylysine (Sigma-Aldrich) on self-assembled neocartilage was investigated. Following a full-factorial study design, constructs were treated with 0.0016 mg/ml copper sulfate and 0.146 mg/ml hydroxylysine either alone or in combination (copper sulfate + hydroxylysine group), or left untreated to serve as controls. Treatments were carried out for the entire culture duration, after which the neotissue was assayed, as described below.

Histology

Portions of constructs were embedded in Histoprep (Fisher Chemical, Vernon Hills, IL, USA) and sliced to 14 μ m. After being fixed in chilled formalin, sections were stained with Picrosirius Red for total collagen and Safranin O/Fast Green for GAG.

Biochemistry

The wet and dry (following lyophilization for 2 d) weights of the construct segments designated for biochemical analysis were determined, after which the samples were digested in papain as described previously (24). A chloramine-T hydroxyproline assay using a Sircol collagen assay standard (Accurate

TABLE 1. Engineered neocartilage's properties at end of 6-wk culture period

Group	Wet weight (mg)	Water content (%)	Thickness (mm)	Diameter (mm)	Cells/construct ($\times 10^6$)
Control	51.9 \pm 2.7 ^a	86.9 \pm 1.9 ^a	1.95 \pm 0.34 ^a	5.94 \pm 0.12	6.08 \pm 3.31 ^b
Hydroxylysine	45.2 \pm 1.2 ^b	86.7 \pm 1.3 ^a	1.45 \pm 0.06 ^b	6.01 \pm 0.16	13.2 \pm 1.84 ^a
Copper sulfate	29.3 \pm 1.8 ^c	83.2 \pm 2.2 ^b	1.20 \pm 0.08 ^b	5.86 \pm 0.21	8.09 \pm 2.31 ^b
Copper sulfate + hydroxylysine	28.9 \pm 1.1 ^c	86.5 \pm 1.3 ^a	1.29 \pm 0.11 ^b	6.01 \pm 0.17	7.25 \pm 4.36 ^b

^{a,b,c}Values marked with different letters within each category are significantly different ($P < 0.05$), with $a > b > c$.

Chemical and Scientific Corp., Westbury, NY, USA) was used to assess for total collagen, while GAG content was measured using a dimethylmethylene blue dye-binding assay kit (Bio-color, Newtownabbey, UK). DNA content was determined using a Picogreen Cell Proliferation Assay Kit (Molecular Probes, Eugene, OR, USA).

High-performance liquid chromatography (HPLC)

Segments of constructs were digested in 800 μ l of 6 N HCl for 18 h at 100°C prior to drying in a vacuum concentrator. Digested, dried samples were then resuspended in 50 μ l of 10 μ M pyridoxine and 2.4 mM homoarginine. After a 5-fold dilution with 0.5% heptafluorobutyric acid (HFBA) in 10% acetonitrile, 10 μ l of each sample was run on the HPLC as described previously in the literature (25). To quantify the cross-link content, PYR standards (Quidel, San Diego, CA, USA) were used.

Compressive testing

Following a previously established protocol, creep indentation testing was used to evaluate the compressive properties of the engineered neocartilage at the end of 6 wk (26). A micrometer was used to measure the thickness of a specimen prior to testing. Samples were then glued to a stainless steel surface and submerged in phosphate buffered saline for 15 min to reach equilibrium. Next, a 0.8-mm-diameter flat, porous indenter tip was applied to the sample by a 0.7-g mass until the sample crept to equilibrium. Finally, a seminumerical, seminumerical, linear biphasic model was used to approximate the aggregate modulus (H_A), permeability, and Poisson's ratio as described previously (26).

Tensile testing

Tensile testing was conducted using an Instron uniaxial testing machine (Model 5565; Instron, Canton, MA, USA). After being cut into dog-bone shapes, sample thickness and width were measured *via* ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA). Samples were then glued at either extremity to paper strips, which were loaded into the Instron grips. A pull to failure test was then run at a strain rate of 1% of the gauge length (measured as the distance between the glued ends of the dog bone) per second until sample failure. The Young's modulus (E_y) of the sample was calculated as the slope of the linear portion of the resulting stress *vs.* strain curve, while the ultimate tensile strength (UTS) was measured as the maximum stress reached during testing.

Statistics

To test the hypothesis that medium supplemented with copper sulfate and hydroxylysine would improve the biomechanical properties of engineered articular cartilage, a 1-way

ANOVA ($n=6$ /group) was used. When the 1-way ANOVA showed significance ($P < 0.05$), a Fisher's *post hoc* test was applied. Data that had a positive interaction on an additive scale and resulted in a combined group that was greater than the addition of the 2 singular treatments were determined to be synergistic. Data are reported as means \pm SD.

RESULTS

Gross morphology and histology

Table 1 describes the morphological characteristics of the neocartilage (wet weight, water content, thickness, diameter, and cell content). While constructs from all groups displayed uniform diameter, significant differences were detected in terms of their thickness, wet weight, and water content. Controls presented with significantly greater thickness and wet weight over all treated groups. The copper sulfate group displayed lower water content compared to all other groups. When applied alone, hydroxylysine promoted cell proliferation in the engineered tissue. No significant differences were found to exist among groups in terms of neocartilage morphology, with all constructs maintaining a smooth, flat, disc-shaped appearance (Table 1 and Fig. 2). Histologically, all constructs

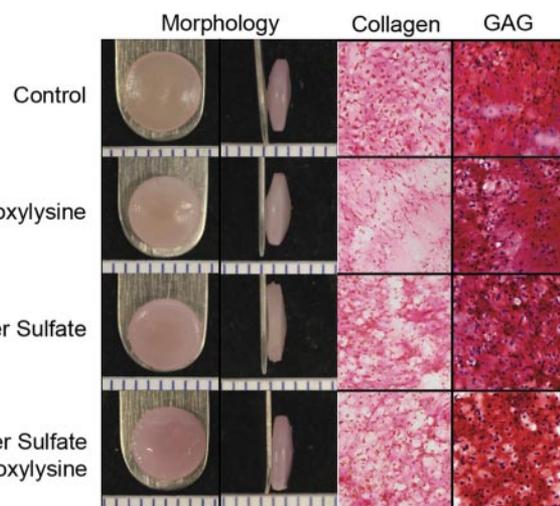


Figure 2. Gross morphology and histology of self-assembled neocartilage at the end of the 6-wk culture period. Both control and treated constructs formed uniform neocartilage without physical abnormalities. Constructs stained positive for Safranin-O/Fast Green staining for GAG and Picrosirius Red staining for collagen in all groups.

stained positive for both collagen and GAG, as displayed in Fig. 2.

Biochemistry

Collagen content normalized to construct wet weight (Col/WW) is presented in Fig. 3A. Percentages of Col/WW were 1.10 ± 0.14 , 0.77 ± 0.08 , 0.98 ± 0.24 , and 0.75 ± 0.12 for control, hydroxylysine, copper sulfate, and copper sulfate + hydroxylysine, respectively. While Col/WW was significantly decreased in all hydroxylysine-treated groups compared to controls, no significant differences were detected between the copper sulfate group and controls. GAG content normalized to construct wet weight (GAG/WW) found the hydroxylysine group to have significantly higher GAG levels than all other groups (Fig. 3B). No significant differences were detected between controls and all copper sulfate-treated groups. Percentages of GAG/WW were 3.2 ± 0.3 , 5.4 ± 1.1 , 3.1 ± 0.3 , and 4.9 ± 1.0 for control, hydroxylysine, copper sulfate, and copper sulfate + hydroxylysine, respectively. However, when GAG was normalized to cell content of the constructs, the hydroxylysine group had significantly lower amount of GAG content per cell, suggesting a decreased synthetic activity of the cells in this group.

HPLC was used to measure the amount of PYR cross-links formed in the engineered neotissue (Fig. 3). When the cross-linked PYR content normalized to construct wet weight (PYR/WW) was analyzed, both treatments containing copper sulfate were found to have significantly greater numbers of cross-links compared to controls (Fig. 3C). With a value of 9.9 ± 5.5 nmol/g, the copper sulfate + hydroxylysine treatment resulted in a synergistic 1016% increase over control values, which were measured at 0.9 ± 0.8 nmol/g. The hydroxylysine and copper sulfate groups, on the other hand, were found to have 3.1 ± 1.5 and 4.9 ± 0.9 nmol/g, respectively. When the cross-linked PYR content normalized to construct collagen content (PYR/Col) was analyzed, similar trends were detected (Fig. 3D). Values of PYR/Col were 0.08 ± 0.07 , 0.39 ± 0.17 , 0.49 ± 0.15 , and 1.61 ± 0.15 nmol/mg for control, hydroxylysine, copper sulfate, and copper sulfate + hydroxylysine, respectively.

Biomechanics

To understand the influence of copper sulfate and hydroxylysine on the biomechanical properties of engineered articular cartilage, biomechanical testing was conducted (Fig. 4). Creep indentation testing found

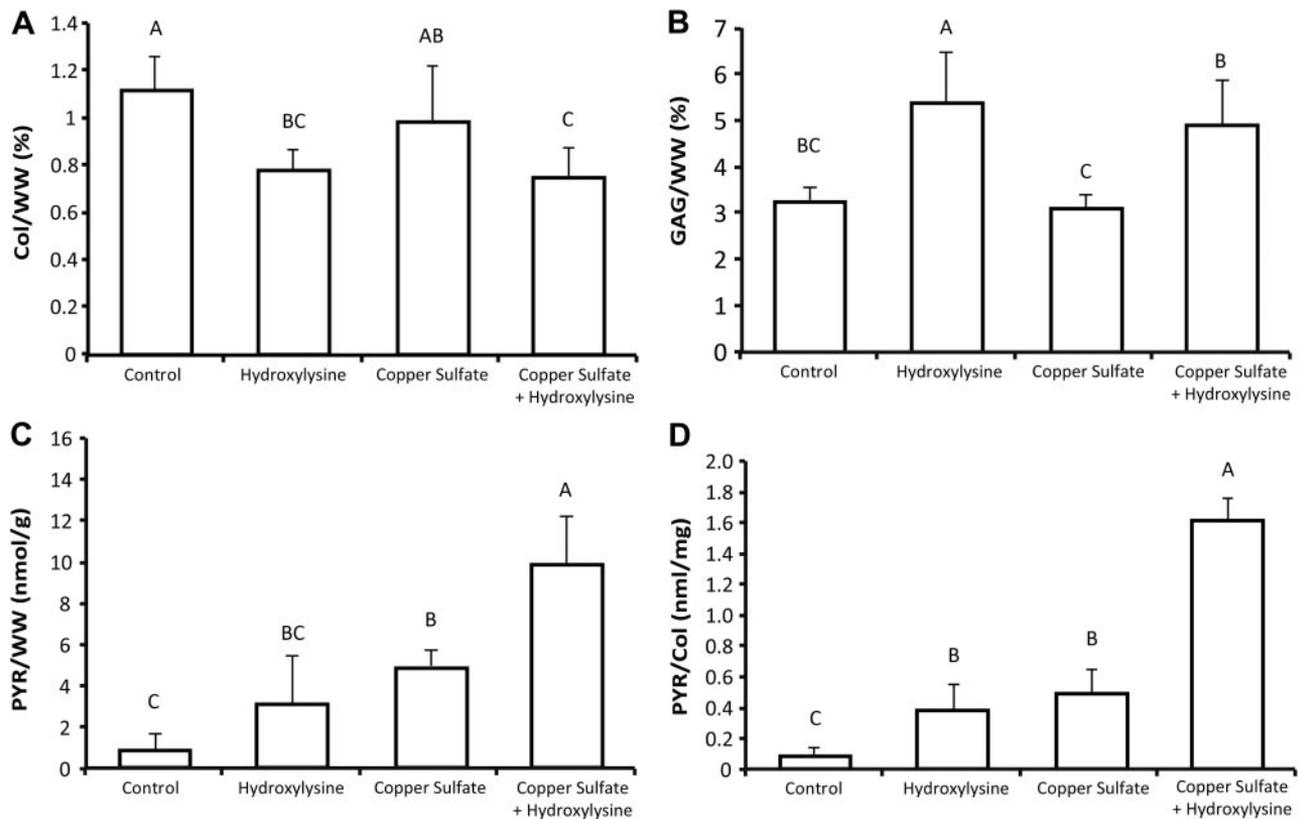
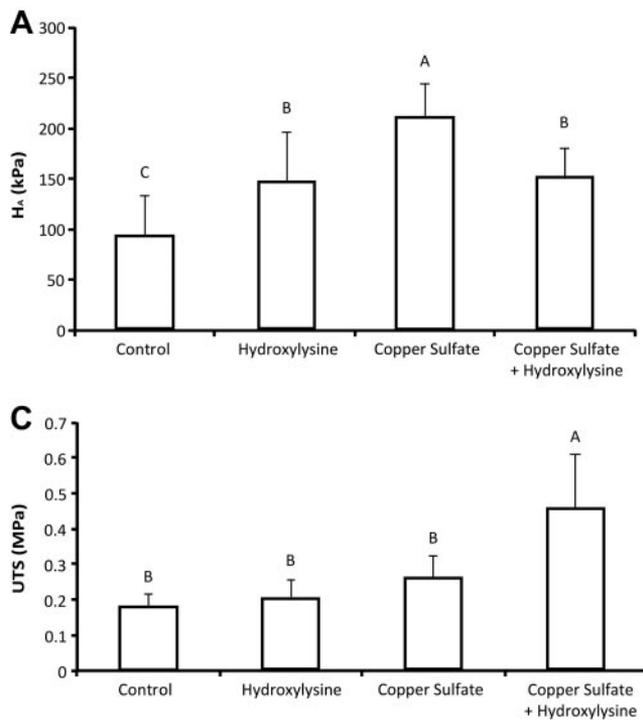


Figure 3. Biochemical properties of tissue-engineered neocartilage: Col/WW (A), GAG/WW (B), PYR/WW (C), and PYR/Col (D). Col/WW was significantly decreased in all hydroxylysine-treated groups compared to controls. No significant differences were detected between the copper sulfate group and controls. In terms of GAG/WW, no significant differences were detected between controls and the copper sulfate-treated groups, while hydroxylysine significantly increased the amount of GAG/WW over controls. HPLC found all copper sulfate-treated groups to have significantly greater amounts of PYR/WW compared to controls, with a synergistic increase measured in the copper sulfate + hydroxylysine group. Similar trends were detected when the amount of PYR cross-links were normalized to construct collagen content. Bars represent means \pm sd; groups not connected by the same letter are statistically different ($P < 0.05$).



UTS, however, found only the copper sulfate + hydroxylysine group to be significantly higher than all groups. Bars represent means \pm SD; groups not connected by the same letter are statistically different ($P < 0.05$).

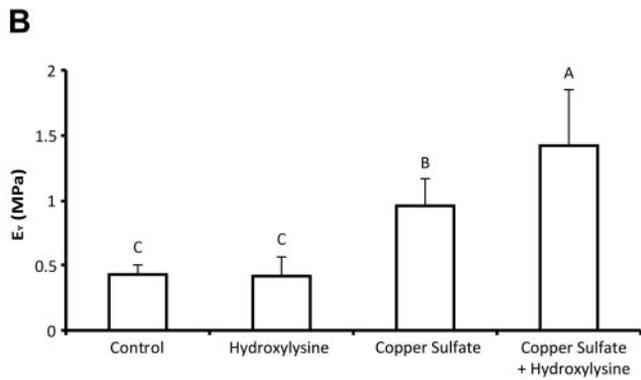


Figure 4. Biomechanical properties of self-assembled neocartilage: H_A (A), E_Y (B), and UTS (C). Creep indentation testing found that all treatments significantly increased the compressive modulus of the engineered neocartilage. The copper sulfate group presented with the highest H_A and resulted in a 123% significant increase over controls. Both the copper sulfate and copper sulfate + hydroxylysine groups significantly increased the tensile modulus of the constructs over the control and hydroxylysine groups. The copper sulfate + hydroxylysine group presented with the highest tensile modulus among all groups, resulting in a significant 3.3-fold increase over controls. Results for the

that all treatments significantly increased the H_A of the engineered neocartilage over controls (Fig. 4A). H_A values were 95 ± 41 , 146 ± 50 , 211 ± 35 , and 153 ± 30 kPa for control, hydroxylysine, copper sulfate, and copper sulfate + hydroxylysine, respectively. The copper sulfate group presented with the highest H_A and resulted in a 123% significant increase over controls. Permeability values were 3.07 ± 0.83 , 4.05 ± 2.77 , 4.06 ± 2.66 , and $1.99 \pm 2.04 \times 10^{-15} \text{ m}^4 \cdot \text{N}^{-1} \cdot \text{s}^{-1}$, for control, hydroxylysine, copper sulfate, and copper sulfate + hydroxylysine, respectively, signifying no differences among the groups. Poisson's ratio, on the other hand, was significantly lower in the copper sulfate-treated group; values were 0.24 ± 0.06 , 0.27 ± 0.05 , 0.13 ± 0.12 , and 0.14 ± 0.11 , for control, hydroxylysine, copper sulfate, and copper sulfate + hydroxylysine, respectively.

Tensile testing found that the copper sulfate and copper sulfate + hydroxylysine groups, with values of 1.0 ± 0.2 and 1.3 ± 0.5 MPa, respectively, had significantly greater E_Y values than either the control or hydroxylysine groups (Fig. 4B). Specifically, the copper sulfate + hydroxylysine group presented with the highest tensile modulus among all groups, resulting in a significant 3.3-fold increase over controls. No significant differences were detected between the control and hydroxylysine groups, with values of 0.4 ± 0.7 and 0.4 ± 0.1 MPa, respectively. In terms of the UTS, the copper sulfate + hydroxylysine-treated group exhibited a higher value than all other groups (Fig. 4C). Overall, the UTS values of the engineered neotissue were 0.18 ± 0.03 , 0.20 ± 0.05 , 0.26 ± 0.06 , and 0.46 ± 0.15 MPa for the control, hydroxylysine, copper sulfate, and copper sulfate + hydroxylysine, respectively.

DISCUSSION

The purpose of this study was to enhance collagen cross-linking in engineered articular cartilage and therefore improve the tissue's biomechanical properties. To carry out this goal, 2 agents with important *in vivo* roles in collagen cross-link formation were administered to the engineered tissue: copper sulfate and hydroxylysine. It was hypothesized that supplementing chondrogenic medium with both copper sulfate and hydroxylysine would additively or synergistically increase the quantity of LOX-mediated collagen cross-links in self-assembled articular cartilage, strengthening the collagen network and, thus, improving the tensile properties of the neotissue. The hypothesis was confirmed *via* a full-factorial study design, demonstrating that the treatments enhanced both collagen cross-link content and biomechanical properties. Specifically, the combination of copper sulfate and hydroxylysine synergistically enhanced the PYR cross-links of the neotissue by 1016% over controls, manifesting in a tensile modulus within this group that was 3.3-fold larger than that of the control. In terms of the compressive properties, all treatments significantly increased the H_A over controls. The copper sulfate treatment displayed the highest compressive modulus and resulted in an 123% increase over controls. Novel contributions of this study include demonstrating that exogenous application of both copper sulfate and hydroxylysine increase the number of collagen cross-links in engineered cartilage; these treatments enhance both the tensile and compressive properties of the tissue; and, when applied alone, hydroxylysine promotes cell proliferation within the 3-dimensional culture environment provided by self-assembly.

In addition to the novel contributions listed above, the treatments examined in this study resulted in other biochemical and morphological findings, which, when considered together, help explain the most significant findings of this study. For instance, all treated constructs presented with significantly lower thickness and higher pyridinoline content, suggesting that collagen cross-linking prevents expansive cartilage growth *in vitro* (27). Further, concerning the proliferative effect of hydroxylysine, past work has shown that proliferative activity minimizes the synthetic activity of chondrocytes (28). This may provide a plausible explanation of why these highly cellular constructs presented with significantly lower collagen and GAG (when normalized to the cell content), greater water content, and concomitantly lower biomechanical properties (6, 29). It should be noted that no significant differences in cell number were found between control and copper sulfate + hydroxylysine-treated constructs. Possibly, the highly cross-linked collagen network within the combination-treated constructs inhibited the proliferative activity of hydroxylysine due to strong cell-ECM interactions (30). Another possibility is that with addition of copper sulfate, LOX was activated even further, thus modifying and sequestering the proliferative effect of hydroxylysine. While this mechanism remains unverified, the benefit of copper curtailing hydroxylysine's proliferative effect is that the combined treatment group did not suffer from loss of GAG and collagen when normalized to cell content, unlike the hydroxylysine treatment group. This finding, in combination with the synergistic increase in pyridinoline cross-links within the copper sulfate + hydroxylysine-treated group, may provide an explanation of why the combined treatment resulted in constructs having higher tensile properties than all other treatments.

Copper sulfate was chosen in this study for its potential to enhance collagen cross-linking in engineered tissue based on its principal role in forming LOX-mediated PYR cross-links *in vivo*. The importance of copper sulfate has been demonstrated in various developmental studies. Such work has linked dietary copper deficiencies in mammals with abnormal skeletal and vascular development (31). Further, LOX activity has been correlated with dietary copper, indicating that abnormal development is likely due to the inability of LOX to form stabilizing cross-links in copper-deficient animals (32). The effect of copper sulfate on cartilage has also been studied *in vitro*. One such study found the addition of copper in the range 0.001–0.034 mg/ml to up-regulate collagen synthesis in cultured human articular chondrocytes by ~25%, while proteoglycan levels remained unchanged (33). In a separate study, 0.002–0.319 mg/ml copper sulfate was administered to porcine cartilage explants grown *in vitro*. At concentrations of <0.016 mg/ml, the copper sulfate was found to have no effect on proteoglycan synthesis. However, at concentrations of >0.04 mg/ml, copper sulfate was found to cause a dose-dependent increase of this ECM protein (34). Together, these studies signify the important effects of copper sulfate for the development of a stabilized collagen matrix in a dose-dependent manner.

While several investigations have taken place showing

the important effect of copper sulfate from a developmental standpoint as well as on native cartilage explants and chondrocytes, no studies have thus far investigated the potential for copper sulfate to enhance engineered cartilage. It should be reemphasized that the basic medium formulations used in most cartilage tissue engineering endeavors, such as DMEM, do not contain copper sulfate, limiting LOX-mediated collagen cross-linking. Limited development of cross-links could affect the biomechanical integrity of the forming collagen network, leading to the relatively low biomechanical properties observed in engineered tissues. Reflecting on past work in which chondrocytes were treated with copper sulfate, the present study supplemented self-assembled articular cartilage with 0.0016 mg/ml copper sulfate. Results found increased levels of collagen cross-links (444% increase over controls) to correspond with significant, 2.2-fold changes in both the tensile and compressive properties compared to controls. Although this study did not directly assess the activation of LOX by copper sulfate, other studies have amply verified the dependence of LOX on the presence of copper (18–20). Overall, the results of this study shed light onto the potential to improve engineered cartilage *via* the enhancement of PYR collagen cross-linking as promoted by copper sulfate supplementation.

The concentration of copper sulfate used in this study to promote PYR collagen cross-linking did not affect the viability of the cells within the engineered cartilage. Past work has found free circulating copper sulfate ions at physiological concentrations to lead to hydroxyl radical formation *via* reduction of Cu^{2+} by either superoxides or ascorbic acid, the latter of which is an important medium component for engineered tissues (35, 36). Therefore, when supplemented at too high of a concentration, copper ions can cause cellular toxicity. Results of this study found that the relatively low concentration of copper ions supplemented in the medium did not have a toxic effect on the cells, as the total cell number per construct did not significantly change in those subjected to copper sulfate. These results are similar to those found in a study on engineered arteries, where similar levels of copper sulfate were used with no toxic side effects (37). Another possible detriment of using copper sulfate is that, in its ionic form, it has been shown to reduce the half-life of ascorbic acid (38). With ascorbic acid being crucial for stimulating collagen production for *in vitro* tissue culture, decreasing the longevity of ascorbic acid could negatively impact collagen synthesis. Results, however, found that copper sulfate did not significantly change the amount of collagen in the engineered tissue, providing further validation that the amount used in this study is not deleterious to the formation of engineered articular cartilage.

Previously, only one study investigated the effects of copper sulfate on modifying the biochemical and structural properties of engineered tissue. In that case, engineered arteries were supplemented with either 0.2 or 0.4 g copper sulfate/ml for the entire 7-wk culture period, or treated with 0.2 g copper sulfate/ml for the first 4 wk, followed by 0.4 g copper sulfate/ml for the remaining 3 wk of the culture

period. Results found the latter regimen of copper sulfate supplementation to increase the number of cross-links in the tissue to 2.5 mol PYR/mol collagen, compared to 1 mol PYR/mol collagen in tissue treated with 0.2 or 0.4 g copper sulfate/ml supplemented for the entire duration of culture. Despite the significant cross-link increase, no significant differences in the maximum elastic modulus or burst pressure of the engineered arteries were found. The authors attributed this lack of improvement in tensile properties to the low levels of collagen fibrils present in their tissue (37). In contrast, the present study showed that copper sulfate was able to significantly increase both the number of cross-links as well as the tensile properties of the tissue. Thus, as previously proposed (39), the tensile strength of a collagen matrix does not only depend on collagen cross-links, but also on the interplay within the collagen network among parameters such as fibril density, diameter, and orientation.

In addition to copper sulfate activation of LOX to catalyze the formation of PYR cross-links, proper incorporation of lysine in both its normal and hydroxylated forms into the collagen fibrils is essential to cross-link formation (40). Dietary supplementation of radiolabeled hydroxylysine has shown that hydroxylysine is not incorporated into skin collagen, as this amino acid can be readily processed by gut bacteria (41, 42). In contrast, whether hydroxylysine made directly available to chondrocytes can be incorporated into collagen is currently unknown. With respect to articular cartilage, dietary supplementation *vs.* direct medium addition of chondroitin sulfate can yield profoundly different effects (43–45), and the same might be the case for hydroxylysine. With regards to the incorporation of amino acids in their hydroxylated forms into collagen fibrils, only a limited number of studies currently exist. Exogenously administered hydroxylysine is capable of being incorporated into proteins *in vitro* by prokaryotic microorganisms (46, 47), which share a similar pathway to mammalian cells to generate protein out of amino acids (48). Work using chick embryos has shown that exogenous hydroxyproline can be incorporated into collagen molecules (49), suggesting that hydroxylysine might likewise be incorporated into collagen in this study.

Although the way chondrocytes use exogenously applied hydroxylysine remains unknown, the present study has found the combination of hydroxylysine and copper sulfate to synergistically increase the PYR content of the neotissue >10-fold. Physiological PYR levels in 1- to 3-wk-old bovine articular cartilage have been reported at 95.5 ± 29.4 nmol/g (9). Should the generated cartilage be implanted, cross-links within the implant are expected to continue to increase by utilizing the copper that is naturally available *in vivo*. While the combined treatment promoted a synergistic increase in the PYR content of the neotissue over the individual treatments, a significant, although not synergistic, enhancement was observed in the tensile properties of the constructs of the same group. This suggests that a nonlinear correlation may exist between collagen cross-links and the tensile properties of engineered tissues. There is also the possibility of cross-links form-

ing between monomeric hydroxylysines outside of the collagen fibers. Future studies will therefore be important to help elucidate how chondrocytes interact with exogenously applied hydroxylysine.

Aside from the physical architecture of the collagen matrix, it has been shown that the ability of LOX to generate collagen cross-links is dependent on the monomeric or polymeric state of the collagen fibers themselves. LOX activity has been found to be dependent on the molecular chain length of the substrate (50). Specifically, LOX activity was much higher on intact collagen fibrils than on monomeric collagen. It was suggested that LOX acts on collagen fibril intermediates, binding to and stabilizing growing, as opposed to mature, fibrils. Therefore, it can be seen that recapitulating the *in vivo* formation of collagen fibrils is crucial for enzyme-mediated collagen cross-linking. This suggests that perhaps the way collagen fibrilogenesis occurs *in vitro* does not provide LOX with the intermediate collagen fiber substrate it requires, decreasing its ability to correctly form PYR cross-links in engineered tissue. Future work toward better understanding collagen matrix formation in engineered tissues and how it compares to that of native tissues may help explain why engineered tissues have comparatively inferior biomechanical properties. Such studies will help provide insight toward developing more mechanically functional collagen networks in engineered tissues, which will, in turn, help advance the field toward generating clinically relevant replacement and reparative tissues.

While this study hypothesized that enhanced collagen cross-linking would increase the tensile properties of engineered articular cartilage, results found the copper sulfate treatment to also significantly enhance the compressive properties of the tissue by 123% over control values, as well as over other treated groups. Interestingly, results found the hydroxylysine containing groups to have significantly increased GAG/WW over controls; GAG levels in copper sulfate-treated constructs, however, were not significantly different than controls. This result may at first appear counterintuitive, especially when one considers the structure-function paradigm associated with articular cartilage, that tensile properties are associated with collagen content while compressive properties are linked to GAG content (6). Although GAG plays an important role in providing articular cartilage with compressive robustness, recent reports have found collagen density and organization also to be important for the compressive properties of this tissue (7–9). It has been shown that a better collagen network can more effectively immobilize negatively charged proteoglycans, thus increasing the mechanical stability of the network in both tension and compression (51). Therefore, with increased collagen cross-links, it follows that the increased stabilization of the collagen network increases resistance to compressive loading. In addition, the copper sulfate alone treatment significantly decreased the water content of the engineered neotissue. With no significant differences between the thickness and diameter of the copper sulfate-treated constructs compared to all other groups, this suggests that copper sulfate promotes the development of a denser ECM. This is

further corroborated by maintenance of the collagen and GAG content in this group compared to controls. Taken together, copper sulfate appears to yield a functionally superior ECM in engineered neocartilage in terms of construct biomechanical properties. With both tensile and compressive properties improved, copper sulfate offers much promise as a potent agent toward generating clinically relevant repair cartilage.

An additional finding in this study was that exogenous application of hydroxylysine significantly increased the number of cells per construct, 117% over controls. This finding is pertinent to the field of tissue engineering, as a major problem with clinically relevant cell sources is their limited availability. When working with such cell sources, methods to induce *ex vivo* proliferation are therefore necessary to achieve high enough cell numbers to engineer tissues. Unfortunately, past work has found that passaging and expanding cells in monolayer typically leads to a loss of phenotype (52). Results of this study suggest that simply adding hydroxylysine to the medium of engineered neocartilage significantly increases cell numbers. Although the potential of hydroxylysine, as any chemical agent applied to articular chondrocytes, to affect the phenotype of the cells exists, application of hydroxylysine in the combined group resulted in significantly increased mechanical properties and cartilage-like tissue, as confirmed by histology and biochemistry. Thus, results of this study suggest that hydroxylysine did not alter the chondrogenic phenotype. While future work should be carried out to investigate the effects of hydroxylysine as a potent proliferative agent in different 3-dimensional environments, using other cell types, as well as monitoring cellular phenotype following proliferation, this work promises a new avenue to promote the expansion of cells in 3-dimensional culture. Ultimately, this may be an important step toward solving the limited availability of clinically relevant cell sources for tissue engineering applications.

Making up 50–80% of the dry weight of articular cartilage, collagen fibers act as the main mechanical support structure of cartilage, providing the tissue with the means to effectively distribute and withstand high tensile loading within the joint space (53). While offering much promise as an effective remedy for diseased cartilage, engineered cartilage replacements currently do not have the mechanical strength to withstand the high levels of *in vivo* loading. This study focused on a novel approach to enhance the tensile properties of engineered cartilage through reinforcing the collagen network *via* PYR cross-links. Using copper sulfate and hydroxylysine, 2 important agents in the development of such cross-links, this study found the use of both agents to synergistically enhance the number of PYR cross-links in the engineered tissue. Further, both copper sulfate and copper sulfate + hydroxylysine treatments significantly enhanced the tensile properties of the tissue. In addition, all treatments significantly increased the compressive modulus over controls, with the copper sulfate treatment displaying the highest values. Hydroxylysine was also identified as a promising agent to promote cellular proliferation in a 3-dimen-

sional culture environment. Overall, this work speaks to the importance of ensuring proper collagen cross-linking in the generation of robust tissues capable of having a clinical impact. Future work should attempt to understand how hydroxylysine is used by articular chondrocytes, use gene expression assays to investigate whether copper sulfate up-regulates LOX expression, as well as focus on understanding how collagen networks are developed in engineered tissues. Together, such work will aid in the quest to generate clinically relevant regenerative tissues. FJ

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